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(54) METHOD FOR SCREENING CANDIDATE COMPOUNDS FOR DRUG AGAINST TUMOR

(57) Investigation on the frequency of FLT3/ITD found in various blood cancers has revealed that the frequency is high in acute myeloblastic leukemia in particular. Studies on the effects of FLT3/ITD in the blood cell lines revealed that the tyrosine residues in FLT3/ITD is constitutively phosphorylated in these cell lines and that blood cells into which FLT3/ITD is introduced show IL-3 independent proliferation. Moreover, the blood cells into which FLT3/ITD is introduced are found to be capable of forming tumors and inhibit cell differentiation. The inventors have found that it is possible to screen for pharmaceutical compounds against tumors by using inhibition of these FLT3/ITD functions as an index.

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Description

Technical Field

[0001] The present invention relates to a method for screening a candidate compound for a drug against tumors, in particular, blood cancers. More specifically, it relates to a method for screening a compound that inhibits the function of FLT3/ITD in animal cells, including blood cell lines.

Background Art

[0002] FTL3, as well as KIT, FMS and PDGFR, is a protein of a class III receptor tyrosine kinase (RTK), and is presumed to be involved in the hematopoietic system (Rosnet, O. et al., 1991, Genomics 9:380-385; Rosnet, O. et al., 1991, Oncogene, 6:1641-1650; Matthews, W. et al., 1991, Cell, 65: 1143-1152; Rosnet, O. et al., 1993, Blood, 82: 1110-1119). Structurally, RTK has an extracellular region containing five immunoglobulin-like domains, one juxtamembrane region (JM domain), two tyrosine domains (TK1 and TK2) intervened by a kinase insert domain (KI domain), and the C-terminal domain. FLT3 is strongly expressed in the hematopoietic stem cells as well as the brain, placenta and liver (Rosnet, O. et al., 1991, Oncogene 6:1641-1650; Matthews, W. et al., 1991, Cell, 65: 1143-1152; Rosnet, O. et al., 1993, Blood, 82: 1110-1119; Rusten, L.S., 1996, 87: 1317-1325). A ligand for FLT3 (FL) is expressed from stromal cells in the bone marrow, and present in a membrane-bound or soluble form. This ligand stimulates stem cells independently or together with other cytokines (Hannum, C. et al., 1994, Nature, 368: 643-648; McKenna, H. J. et al., 1995, Blood, 86: 3413-3420; Hirayama, F., 1995, Blood, 85: 1762-1768; Lisovsky, M. et al., 1996, Leukemia, 10: 1012-1018). Therefore, the ligand-receptor interaction between FL and FLT3 is thought to play an important role in the hematopoietic system.

[0003] On the other hand, high levels of FLT3 expression are observed in most of the specimens from patients with acute myeloid leukemia (AML) or acute chronic lymphocytic leukemia (ALL). High levels of FLT3 expression are also found in the patients with chronic myeloid leukemia (CML). FL is known to stimulate the proliferation of AML cells more prominently than AML cells (Piacibello, W. et al., 1995, Blood, 86: 4105-4114; Stacchini, A. et al., 1996, Leukemia, 10: 1584-1591; Lisovsky, M. et al., 1996, Blood, 88: 3987-3997; Birg, F. et al., 1992, Blood, 80: 2584-2593, Dehmel, U. et al., 1996, Leukemia, 10: 261-270). This indicates that FLT3 has a function specific to myeloid cells. In several leukemia-lymphoma cell lines, FLT3 and FL are co-expressed (DaSilva, N. et al., 1994, Leukemia, 8: 885-888; Meierhoff, G. 1995, Leukemia, 9: 1368-1372), suggesting their autocrine or paracrine mechanism.

[0004] Mutations in cytokine receptors that occur in the process of tumorigenesis have received attention in recent years. To date, mutations in c-fms and c-kit in human leukemia have been reported (Lowenverg, B. and Touw, I. P., 1993, Blood, 81: 281-292). Murine NIH3T3 cells transfected with a mutant c-fms undergo ligand-independent transformation (Roussel, M. et al., 1988, Cell, 55: 979-988). M-CSF, a ligand for fms, increases cell proliferation only slightly in the most of leukemic patients. Thus, the significance of FMS mutation is still unknown (Lowenberg, B. and Touw, I. P., 1993, Blood, 81: 281-292). KIT and its ligand SCF stimulate proliferation of leukemia and stem cells (Lowenberg, B. and Touw, I. P., 1993, Blood, 81: 281-292; Witte, O., 1990, Cell, 63: 5-6). However, mutations in c-kit gene have been found in the mast cell leukemic cell lines, which mutations have not been fully identified in clinical samples (Tsujimura, T. et al., 1994, Blood, 83: 2619-2626; Kitayama, H., 1996, Blood, 88: 995-1004; Tsujimura, Y. et al., 1996, Blood, 87: 273-283).

[0005] Recently, somatic mutations in FLT3 were found in AML patients (Nakao, M. et al., 1996, Leukemia, 10: 1911-1918). In these mutants, internal tandem duplication (ITD) was found in the region coding for the JM domain of the FLT3 gene. The duplicated sequences predominantly contain exon 11/12 and intron 11, though varying in length in each sample, and they commonly have an extended JM domain which is translatable in a protein due to an extended in-frame open reading frame.

[0006] FLT3 mutations are found in about 20% of AML patients and about 3% of patients with myelodysplasia syndrome (MDS), but not in patients with chronic myeloid leukemia (CML) or lymphocytic blood cancer (Yokota, S. et al., 1997, Leukemia 11: 1605-1609). To the inventors' knowledge, mutant FLT3 genes containing ITD (hereinafter referred to as "FLT3/ITD") are found in some AML patients when the tumor recurs, even though they are not found by the early diagnosis, suggesting that FLT3/ITD is responsible for progression of leukemia. However, the role of FLT3/ITD in the progression of leukemia has not been reported so far.

Disclosure of the Invention

[0007] An objective of the present invention is to elucidate the FLT3/ITD function in blood cancers, such as leukemia, and to provide a screening method for a candidate compound for a drug against tumors, such as blood cancer, using inhibition of the FLT3/ITD function as an index.

[0008] To solve the problems above, the inventors have investigated the functions of FLT3/ITD in the blood cell lines to find that tyrosine residues present in FLT3/ITD are costitutively phosphorylated in the cell lines tested, that blood cells into which FLT3/ITD is introduced proliferate in an IL-3-independent manner, and that syngeneic mice inoculated with those blood cells develop tumors. These findings suggest that IL-3-independent cell proliferation is induced by proliferative signaling of FLT3/ITD mediated by tyrosine-phosphorylation of FLT/ITD, and that this proliferation, in particular, is responsible for progression of tumors in hematopoietic organs, such as acute myeloid leukemia. Based on these observations, the inventors found that inhibition of FLT3/ITD function can be used as an index to screen for a candidate compound for a drug that can be used in the treatment of blood cancers, etc.

[0009] Specifically, the present invention relates to a method for screening a candidate compound for a drug used in the treatment of tumors, using inhibition of FLT3/ITD function in the blood cells as an index. More specifically, the present invention relates to:

(1) a method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

(a) providing animal cells showing cytokine-independent proliferation due to expression of FLT3/ITD,
(b) contacting said cells with a test sample and culturing said cells in the absence of cytokines,
(c) detecting the proliferation of said cells, and
(d) selecting a compound that inhibits the proliferation of said cells;

(2) a method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

(a) providing animal cells showing cytokine-independent proliferation due to expression of FLT3/ITD,
(b) contacting said cells with a test sample and culturing said cells in the absence of cytokines,
(c) detecting phosphorylation of FLT3/ITD in said cells, and
(d) selecting a compound that inhibits the phosphorylation of FLT3/ITD in said cells;

(3) a method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

(a) providing animal cells showing cytokine-independent proliferation due to expression of FLT3/ITD,
(b) inoculating a non-human mammal with said cells to develop tumors,
(c) administering to said non-human mammal a test sample before or after the inoculation with said cells, and detecting the development of the tumor, and
(d) selecting a compound that inhibits the development of the tumor in said non-human mammal;

(4) a method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

(a) providing animal cells in which differentiation-inducing potency is suppressed due to expression of FLT3/ITD,
(b) contacting said cells with a test sample and culturing said cells,
(c) detecting the differentiation-inducing potency of said cells, and
(d) selecting a compound that facilitates differentiation of said cells;

(5) the method according to any one of (1) to (4), wherein said tumor is a blood cancer;
(6) the method according to (5), wherein said blood cancer is acute myeloid leukemia or myelodysplasia syndrome;
(7) the method according to any one of (1) to (3), wherein said cytokine is IL-3;
(8) the method according to any one of (1) to (4), wherein said animal cells are blood cells;
(9) the method according to (8), wherein said blood cells are FDC-P1, 32D, or BaF cells;
(10) the method according to (4), wherein said animal cells are 32D cells; and
(11) a candidate compound for an antitumor drug, wherein said compound can be isolated by the method according to any one of (1) to (10).

[0010] As shown in the working examples, tyrosine-phosphorylation of FLT/ITD was detected in the myeloid cell line, FDC-P1 (ATCC CRL-12103), into which FLT3/ITD was introduced (Example 3). In contrast to the parent cell line FDC-P1, which proliferates IL-3-dependently, FDC-P1 cells into which FLT3/ITD was introduced were found to proliferate IL-3-independently (Example 4). These facts have shown for the first time that tandem duplicate mutation in FLT3 is functionally related to tumorigenesis of blood cells. The inventors have found it possible to inhibit aberrant cell growth and to treat tumors including those found in hematopoietic organs, for example, leukemia, by blocking the FLT3/ITD function as described above.

[0011] One embodiment of the present invention is a method for screening a candidate compound for a drug against tumors, such as blood cancers, using as an index inhibition of the proliferation of animal cells, such as blood cells, in which FLT3/ITD is expressed. Specifically, this method comprises the steps of (a) providing animal cells that proliferate cytokine-independently due to the expression of FLT3/ITD, (b) contacting said cells with a test sample and culturing said cells in the absence of cytokines, (c) detecting the proliferation of said cells, and (d) selecting a compound that inhibits said cell proliferation.

[0012] Another embodiment of the present invention is a method for screening a candidate compound for a drug against tumors, such as blood cancers, using as an index inhibition of tyrosine-phosphorylation of FLT3/ITD in blood cells, etc. Specifically, this method comprises the steps of (a) providing animal cells that proliferates cytokine-independently due to expression of FLT3/ITD, (b) contacting said cells with a test sample and culturing said cells in the absence of cytokines, (c) detecting phosphorylation of FLT3/ITD in said cells, and (d) selecting a compound that inhibits the phosphorylation of FLT3/ITD in said cells.

[0013] Another embodiment of the present invention is a method for screening a candidate compound for a drug against tumors, such as blood cancers, using as an index inhibition of tumorigenesis in animal cells, such as blood cells, in which FLT3/ITD is expressed. Specifically, this method comprises the steps of (a) providing animal cells that proliferate cytokine-independently due to expression of FLT3/ITD, (b) inoculating a non-human mammal with said cells to develop tumors, (c) administering to said non-human mammal a test sample before or after the inoculation with said cells, and detecting the development of the tumor, and (d) selecting a compound that inhibits the development of the tumor in said non-human mammal.

[0014] Yet another embodiment of the present invention is a method for screening a candidate compound for a drug against tumors, such as blood tumors, using as an index differentiation-inducing potency, i.e. an effect that facilitates cell differentiation, of animal cells, such as blood cells, in which FLT3/ITD is expressed. Specifically, this method comprises the steps of (a) providing animal cells in which differentiation-inducing potency is suppressed due to expression of FLT3/ITD, (b) contacting said cells with a test sample and culturing said cells, (c) detecting the differentiation-inducing potency of said cells, and (d) selecting a compound that facilitates differentiation of said cells. Compounds that facilitate cell differentiation include compounds that stimulate the cell differentiation by themselves or together with other cytokines that are known to stimulate the cell differentiation.

[0015] The tumors targeted by drug candidate compounds screened by the method of the present invention include any tumors that are caused by internal tandem duplication (ITD) of FLT3, inter alia, blood cancers, for example, acute myeloid leukemia and myelodysplasia syndrome. In particular, acute myeloid is the most preferable as a target disease.

[0016] Test samples used in the screening include, but are not limited to, purified proteins (including antibodies), expression products from a gene library, a library of synthetic peptides, cell extracts, cell culture supernatant, a library of synthetic low-molecular-weight compounds, oligonucleotides, etc.

[0017] Any animal cell can be used as the cells used for the screening as long as it proliferates cytokine-independently or has suppressed differentiation-inducing potency due to the FLT3/ITD expression. Blood cells (including hematopoietic stem cells) are preferable. Such cells include, for example, FDC-P1 cells (ATCC: CRL-12103), 32D cells (RICKEN (The Institute of Physical and Chemical Research) Cell Bank: RCB 1145), Ba/F3 cells (RICKEN Cell Bank: RCB 0805), DA-3 cells (RICKEN Cell Bank: RCB 1144), all of which show IL3-independent cell proliferation. Among them, in particular, FDC-P1, 32D, and Ba/F3 cells are preferable. Intracellular expression of FLT3/ITD can be carried out by means of a genetic engineering technique well known to those skilled in the art. Any FLT3/ITD can be used for its expression in the cell, as long as it induces the proliferation of blood cells in a cytokine-independent manner. Such FLT3/ITD includes, for example, FLT3/ITD comprising any one of the amino acid sequences of SEQ ID NO: 2, 4, 6 and 8. The FLT3/ITD sequences described in the literatures (Yokota, S. et al. 1997, Leukemia 11: 1605-1609; Kiyoi, H. et al. 1997, Leukemia 11: 1447-1452) can be used in this invention. In addition, FLT3/ITD newly obtained from patients with blood cancer can also be used. FLT3/ITD may be synthesized artificially or derived from cells.

[0018] Test samples can be contacted with cells by the method suitable to the samples used, for example, by the method in which a test sample is added to the cell culture medium or the method in which a test sample is introduced into the cells.

[0019] The followings are specific examples of the screening methods of the present invention, but they are not construed to limit the scope of the present invention.

(i) Wild type FLT3 or FLT3/ITD is inserted into a commercially available expression vector carrying a selectable marker such as a neomycin resistance gene, and the vector is introduced into cells, such as FDC-P1 and 32D cells, with an apparatus, such as Bio-Rad Gene Pulser Cuvettes (Bio-Rad). The cells transfected with FLT3/IED are selected, seeded onto a 24-well plate to approximately 5 x 10^4 cells, and cultured in a CO_2 incubator at 37°C in the presence or absence of a test sample. Two or three days later, viable cells are counted by trypan blue staining, MTT assay, or other assays, to select a compound that inhibits the cell proliferation.

(ii) Wild type FLT3 or FLT3/ITD is inserted into a commercially available expression vector carrying a selectable marker such as a neomycin resistance gene, and the vector is introduced into cells, such as FDC-P1 and 32D cells, with an apparatus, such as Bio-Rad Gene Pulser Cuvettes (Bio-Rad). The cells transfected with FLT3/ITD are selected and seeded onto a 10-cm plate to approximately 1 x 10^6 cells. [γ-32P]ATP is added to the plate. The cells are cultured in the presence or absence of a test sample in a CO_2 incubator at 37°C. Subsequently, the cell extract is prepared and immunoprecipitated with anti-FLT3 antibody and anti-phosphorylated-tyrosine antibody, followed by electrophoresis and autoradiography to detect the radioisotope incorporated in FLT3/ITD. A compound that inhibits tyrosine-phosphorylation of FLT3 can thus be selected.

(iii) Approximately 2 x 10^7 FDC-P1 cells transfected with FLT3/ITD by the method described above in (i) are inoculated subcutaneously to DBA2 mice. FDC-P1 cells were established from this mouse strain. About two weeks after the inoculation, the mice developed tumors. FLT3/ITD expression in the tumors formed was identified in DNA and protein levels (Fig. 3A and B). The tumors were developed without any particular treatment, such as irradiation. That is, the cells, including blood cells, which are transfected with FLT3/ITD, can develop tumors by the action of FLT3/ITD when they are inoculated to non-human mammals, such as syngeneic mice. Therefore, a compound that can inhibit the tumorigenesis can be selected by administering a test sample percutaneously, intravenously or orally before or after the inoculation of the cells, such as blood cells, which are transfected with FLT3/ITD, and examining their effect on the development and elimination of the tumors.

(iv) The inventors have found that 32D cells, for example, are IL3-dependent cells and their differentiation are induced in the presence of G-CSF, and that the G-CSF-induced differentiation is inhibited when FLT3/ITD is introduced into the cells. on the basis of this knowledge, the following method has been developed. Approximately 5 x 10^4 cells of the transformed cells, such as blood cells that are transfected with wild type FLT3 or FLT3/ITD by the method described above in (i) are seeded onto a 24-well plate. Cytospin samples are prepared at various time points after adding a test sample to examine their potential for differentiation by staining with May-Giemsa, peroxidase, esterase, alkaline phosophatase, etc. or by flow cytometry to determine the expression of CD11b, CD13, CD14 and CD33, etc. A compound that promotes the differentiation-inducing potency of the cells can thus be selected.

[0020] To eliminate the effects of nonselective cytotoxic substances in the screening system in which cell proliferation is used as an index, the parent cells are cultured in the presence of IL-3 and a test sample is added to the culture to determine the effects of the test sample on IL-3-dependent cell-proliferation in parallel with the experiment above.

[0021] The compounds that can be isolated through the screening of the present invention may vary in their activities. Examples of the compounds include, for example, those inhibiting the function of FLT3/ITD through the direct action thereon, those indirectly inhibiting the FLT3/ITD function by acting on a molecule that binds to FLT3/ITD or phosphorylated FLT3/ITD (e.g. adapter proteins, such as SHC, Grb2, Cbl, PI3K, RAS-GAP and PLC-γ), those acting on a group of proteins that is involved in the signal transduction pathway from FLT/ITD up to cell proliferation, those acting on a protein that can phosphorylate FLT3/ITD and inhibiting the function thereof, those acting on a group of proteins that is involved in the signal transduction pathway from FLT/ITD to cause its phosphorylation, and those dephosphorylating FTL3/ITD that is constitutively phosphorylated.

[0022] These compounds are candidates for a drug to treat tumors, such as blood cancers, in the development which FLT3/ITD is involved. Among the compounds screened, the compounds that specifically inhibit the FLT3/ITD function but not inhibit the function of wild type FLT3 are preferable as candidates for drugs to specifically treat the diseases mentioned above caused by FLT3/ITD.

[0023] When a compound isolated by the screening method of the present invention is used as a drug, it can be formulated by any pharmaceutical manufacturing methods well known in the art. For example, the drug is combined with a pharmaceutically acceptable carrier or medium (e.g. saline, vegetable oil, suspending agent, surfactant, stabilizer, etc.) and administered to patients. The drug can be administered via various routes, for example, percutaneously, intranasally, transbronchially, intramuscularly, intravenously, or orally, depending on the properties of the compound. Dosage may vary depending on the factors, such as age, weight, condition of the patient and the administration method, but those skilled in the art can properly determine the suitable dosage.

Brief Description of the Drawings

[0024] Fig. 1 shows tyrosine-phosphorylation of FLT3 protein in FDC-P1 cells into which wild type FLT3 or FLT3/ITD has been introduced. The cell extracts were immunoprecipitated with anti-FLT3 antibody (IP: FLT3), resolved by SDS-PAGE, and subjected to Western blotting with anti-phosphorylated-tyrosine antibody (Upper panel, IB: pTyr). The same membrane was further subjected to Western blotting using anti-FLT3 antibody (Lower Panel, IB:FLT3). Lane 1, Mtl;

Lane 2, Mt2; Lane 3, Mt3; and Lane 4, Mt4.

[0025] Fig. 2 shows the proliferation properties of FDC-P1 cells into which wild type FLT3 or one of four FLT3/ITD mutants has been introduced. Each mutant was cultured in the absence of IL-3 and FL (Upper left graph, IL3(-)/FL(-)); in the presence of IL-3 (1 ng/ml) (Upper right graph, IL3(1 ng/ml)/FL(-)); in the presence of FL (50 ng/ml) (Lower left graph, IL-3(-)/FL (50 ng/ml)); and in the presence of both IL-3 and FL (Lower right, IL-3 (1 ng/ml)/FL (50 ng/ml)).

[0026] Fig. 3 shows the presence of FLT3/ITD DNA and the expression of its protein in the tumors formed in DBA2 mice that were subcutaneously inoculated with FDC-P1 cells into which FLT3/ITD was introduced. Panel A shows the DNA bands amplified by PCR and resolved by electrophoresis, indicating the presence of FLT3/ITD DNA in the tumors. Panel B shows the results of Western blotting of the whole cell extracts using anti-FLT antibody, indicating the presence of FLT3/ITD protein in the tumors.

Best Mode for Carrying out the Invention

[0027] The present invention will be explained in detail below with reference to examples, but is not to be construed as being limited thereto. Unless otherwise mentioned, "FLT3" used herein represents mutant FLT3 including FLT3/ITD as well as wild type FLT3. The term "aberrant FLT3" refers to any abnormalities of FLT3 including not only expression of mutant to FLT3 but also overexpression of wild type FLT3.

Example 1. Detection of FLT3/ITD in leukemia cells

[0028] High molecular weight DNA was isolated from leukemia cells, and a DNA fragment containing the JM domain of the FLT3 protein was amplified by PCR according to the method described in Kiyoi, H., Leukemia 11: 1447-1452, 1997. The bands that differed from the band of the wild-type in size were excised from agarose gel and purified with Qiaex gel extraction kit (Qiagen), followed by cloning into pMOSBlue T vector (Amersham) according to the manufacturer's instruction. Ten colonies of the recombinants were cultured on the LB medium, and the plasmid DNA was prepared with QIAprep spin plasmid miniprep kit (QIAGEN). Nucleotide sequences of these clones were confirmed by sequencing. Expression of the FLT3 mRNA was confirmed by RT-PCR according to the method described in Kiyoi, H., Leukemia 11: 1447-1452, 1997. The bands that differed from the band of the wild type in size were cloned according to the method described above, and their nucleotide sequences were confirmed by sequencing.

[0029] The results are summarized in Table 1 showing the frequency of FLT3/ITD within various blood cancers (the number of cases in which FLT3/ITD was detected/the total number of cases tested).

Table 1

Diagnosis	Frequency
ALL	0/48
ATL	0/14
CLL	0/15
ET	0/3
ML	0/16
MM	0/38
Histiocytosis	0/1
CML-BC	0/13
CMMoL	0/17
MDS	1/15

Diagnosis	Frequency
AML(total)	35/221
M0	0/2
M1	5/18
M2	4/29
M3	16/124
M4	6/24
M5	4/20
M6	0/1
M7	0/3

[0030] It was confirmed that among various blood cancers, FLT3/ITD is specifically found in AML; its percent frequency was so high as about 20%. FLT3/ITD was also found in MDS, but its percent frequency was so low as about 3%.

Example 2. Introduction of an FLT3/ITD expression vector into blood cells

[0031] Total RNA was extracted from leukemia cells and was used for cDNA synthesis. The cDNA synthesized was used as a template to amplify the MunI-EcoRV fragment, which contains the tandem repeat region found in mutants

FTL3 cDNA, by RT-PCR. MunI-F primer (SEQ ID NO: 11/ 5'-CAACAATTGGTGTTTGTCTCCTCTT-3') and EcRV-R primer (SEQ ID NO: 12/ 5'-CATGATATCTCGAGCCAATCCAAAG-3') were used for the amplification. The amplified fragments were cleaved with MunI and EcoRV (Boehringer-Mannheim-Yamanouchi), resolved on agarose gel, and purified according to the aforementioned method. Expression vector pCDHF3 (a gift from Dr. Olivier Rosnet), which carries a full-length wild type FLT3 cDNA (Rosnet, O. et al., Blood 82:1110-1119; Accession No. S64785), was cleaved with MunI and EcoRV, and the purified FLT3/ITD fragment was inserted into the vector. Four mutants of FLT3/ITD (Mtl, Mt2, Mt3 and Mt4) were used. Nucleotide sequences of the mutated regions of Mtl to Mt4 are shown as SEQ ID NOs. 1, 3, 5 and 7, and their amino acid sequences are shown as SEQ ID NOs. 2, 4, 6 and 8, respectively. Expression vectors for Mt1 to Mt4 were transfected into the blood cells.

[0032] . FLT3/ITD expression plasmids thus obtained were introduced into blood cells as follows. The cells were co-transfected with a 10:1 mixture of any one of the expression plasmids and pBabe-neo vector (Nucleic Acids Res., 18: 3587-3596, 1990) by using Bio-Rad Gene Pulser Cuvettes (Bio-Rad; 300 V, 960 μF), and selected with 800 ng/ml neomycin. After cloning, FLT3 expressions of the cloned cells were verified by FACS and Western blotting to establish the transfected clones.

[0033] FLT3/ITD genes were introduced into a myeloid cell line, FDC-P1 cells.

Example 3. Tyrosine-phosphorylation of the FLT3 molecules in the transfectants

[0034] (1) The transfectants were cultured in RPMI 1640 medium supplemented with 10% FCS (GIBCO), and then centrifuged at 1000 rpm for 5 minutes to recover 2 x 10^7 cells. The cell pellets were washed with PBS, dissolved in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, Nonidet P-40, 50 mM NaF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM Na_3VO_4, 50 mM Na_2MoO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF)), allowed to stand for 1 hour at 4°C, then centrifuged at 15000 rpm for 30 minutes. Rabbit anti-human FLT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the supernatants and the mixture was stirred for 2 hours at 4°C. After adding Protein A/G Plus agarose (Santa Cruz), the resulting mixture was stirred for 2 hours at 4°C and washed with the lysis buffer three times. The pellets were then dissolved in sample loading buffer (0.125 M Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS), subjected to SDS-PAGE, transferred onto Immobilon PVDF membrane (Millipore), and reacted with anti-phosphorylated tyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY, USA). Following the reaction, the bands were detected using the ECL system (Amersham). To confirm the presence of FLT3 protein, the same membrane was incubated in stopping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) for 30 minutes at 70°C, and then reacted with rabbit anti-human FLT3 antibody.

[0035] The results for FDC-P1 cells are shown in Fig. 1. As shown in this figure, all the FLT3/ITDs introduced into FDC-P1 cells were phosophorylated at the tyrosine residues, indicating that a signaling pathway for tyrosine-phosphorylation of FLT3/ITD is constitutively activated. On the contrary, wild type FLT3 protein was not phosphorylated in the absence of FL.

[0036] The same result was obtained for 32D cells.

Example 4. Proliferation properties of FLT3/ITD-introduced cells

[0037] FDC-P1 cells (5 x 10^4 cells) into which wild type FLT3 (wt) or FLT3/ITD (Mt1 to Mt4) was introduced were cultured in the four media indicated below on a 24-well plate at 37°C in a CO_2 incubator.

1. RPMI1640 supplemented with 10% FCS alone (Fig. 2, upper left graph);
2. RPMI1640 supplemented with 10% FCS + 1 ng/ml mouse IL-3 (Genzyme) (Fig. 2, upper right graph);
3. RPMI1640 supplemented with 10% FCS + 50 ng/ml human FL (Purotech) (Fig. 2, lower left graph);
4. RPMI1640 supplemented with 10% FCS + 1 ng/ml mouse IL-3 + 50 ng/ml human FL (Fig. 2. lower right graph).

[0038] The cells were stained with trypan blue and the viable cells were counted at 24-hour intervals for 4 days to determine the proliferation potency of each cell line. The results are shown in Fig. 2. As shown in the figure, the cells into which wild type FLT3 is introduced were not able to proliferate in the absence of IL-3, and this IL-3 dependency did not alter even after FL, a ligand for FLT3, was added to the medium. Also, synergistic effect of IL-3 and FL was not observed. In contrast, the cells into which FLT3/ITD was introduced proliferated at an equivalent level irrespective of the presence of IL-3, and their proliferation rate was significantly higher than that of the cells into which wild type FLT3 is introduced in the presence IL-3. Also, neither proliferation promoting effect nor synergistic effect of IL-3 and FL were found in these cells.

[0039] These data reveal that FLT3/ITD activates intracellular signaling pathways and triggers cytokine-independent proliferation in the myeloid cells.

[0040] The same results were obtained for 32D cells.

Industrial Applicability

[0041] The present invention provides a method for screening candidate compounds for drug against tumors, such as blood cancers, using inhibition of FLT3/ITD function as an index. Compounds to be isolated by the method of the present invention could have an inhibitory effect on aberrant proliferation of cells, such as blood cells, caused by FLT3/ITD expression found in blood cancers, in particular, acute myeloid leukemia, and can thus be utilized for development of pharmaceuticals for these diseases.

SEQUENCE LISTING

<110> CHUGAI SEIYAKU KABUSHIKI KAISHA

<120> METHOD FOR SCREENING CANDIDATE COMPOUNDS FOR ANTITUMOR DRUG

<130> F 1248 EP

<150> JP 1998-233729
<151> 1998-08-20

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tca gat aat gag tac ttc tac gtt gat ttc aga gaa tat gaa tat gat   96
Ser Asp Asn Glu Tyr Phe Tyr Val Asp Phe Arg Glu Tyr Glu Tyr Asp
            20                  25                  30

ctc aaa agc tcc tca gat aat gag tac ttc tac gtt gat ttc aga gaa   144
Leu Lys Ser Ser Ser Asp Asn Glu Tyr Phe Tyr Val Asp Phe Arg Glu
        35                  40                  45

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65                  70                  75                  80

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Ser Asp Asn Glu Tyr Phe Tyr Val Asp Phe Arg Glu Tyr Asp Glu Tyr
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                    20                  25                  30

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            35                  40                  45

cca aga gaa aat tta gag ttt ggg aag gta cta gga tca ggt gct ttt   192
Pro Arg Glu Asn Leu Glu Phe Gly Lys Val Leu Gly Ser Gly Ala Phe
     50                  55                  60

gga aaa gtg atg aac gca aca gct tat gga att agc aaa aca gga gtc   240
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Pro Arg Glu Asn Leu Glu Phe Gly Lys Val Leu Gly Ser Gly Ala Phe
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Claims

1. A method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

   (a) providing animal cells showing cytokine-independent proliferation due to expression of FLT3/ITD;
   (b) contacting said cells with a test sample and culturing said cells in the absence of cytokines;
   (c) detecting the proliferation of said cells; and
   (d) selecting a compound that inhibits the proliferation of said cells.

2. A method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

   (a) providing animal cells showing cytokine-independent proliferation due to expression of FLT3/ITD;
   (b) contacting said cells with a test sample and culturing said cells in the absence of cytokines;
   (c) detecting phosphorylation of FLT3/ITD in said cells; and
   (d) selecting a compound that inhibits the phosphorylation of FLT3/ITD in said cells.

3. A method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

   (a) providing animal cells showing cytokine-independent proliferation due to expression of FLT3/ITD;
   (b) inoculating a non-human mammal with said cells to develop tumors;
   (c) administering to said non-human mammal a test sample before or after the inoculation with said cells, and detecting the development of the tumor; and
   (d) selecting a compound that inhibits the development of the tumor in said non-human mammal.

4. A method for screening a candidate compound for an antitumor drug, said method comprising the steps of:

   (a) providing animal cells in which differentiation-inducing potency is suppressed due to expression of FLT3/ITD;
   (b) contacting said cells with a test sample and culturing said cells;
   (c) detecting the differentiation-inducing potency of said cells; and
   (d) selecting a compound that facilitates differentiation of said cells.

5. The method according to any one of claims 1 to 4, wherein said tumor is a blood cancer.

6. The method according to claim 5, wherein said blood cancer is acute myeloid leukemia or myelodysplasia syndrome.

7. The method according to any one of claims 1 to 3, wherein said cytokine is IL-3.

8. The method according to any one of claims 1 to 4, wherein said animal cells are blood cells.

9. The method according to claim 8, wherein said blood cells are FDC-P1, 32D, or BaF cells.

10. The method according to claim 4, wherein said animal cells are 32D cells.

11. A candidate compound for an antitumor drug, wherein said compound can be isolated by a method according to any one of claims 1 to 10.

Fig. 1

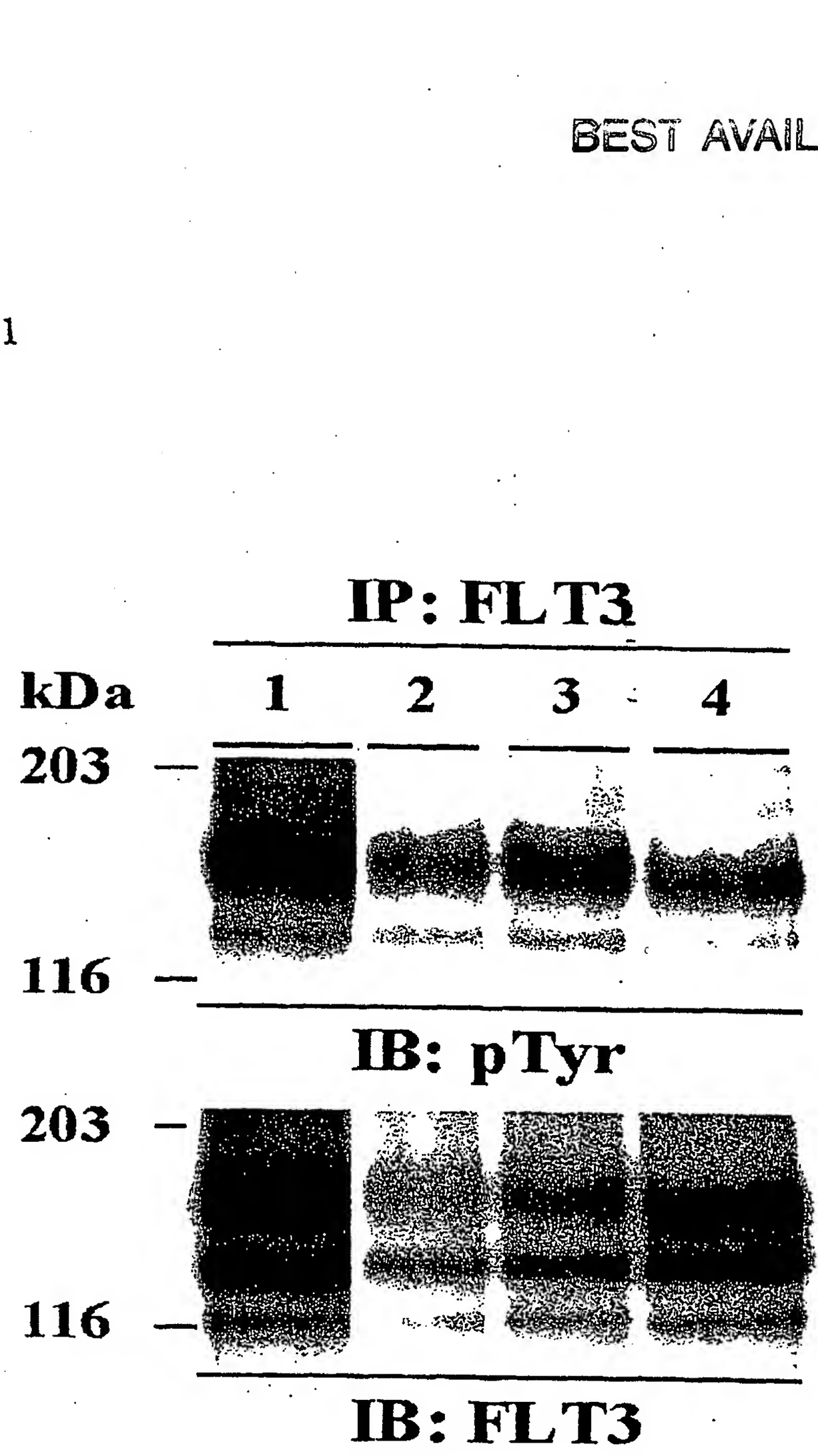


Fig. 2

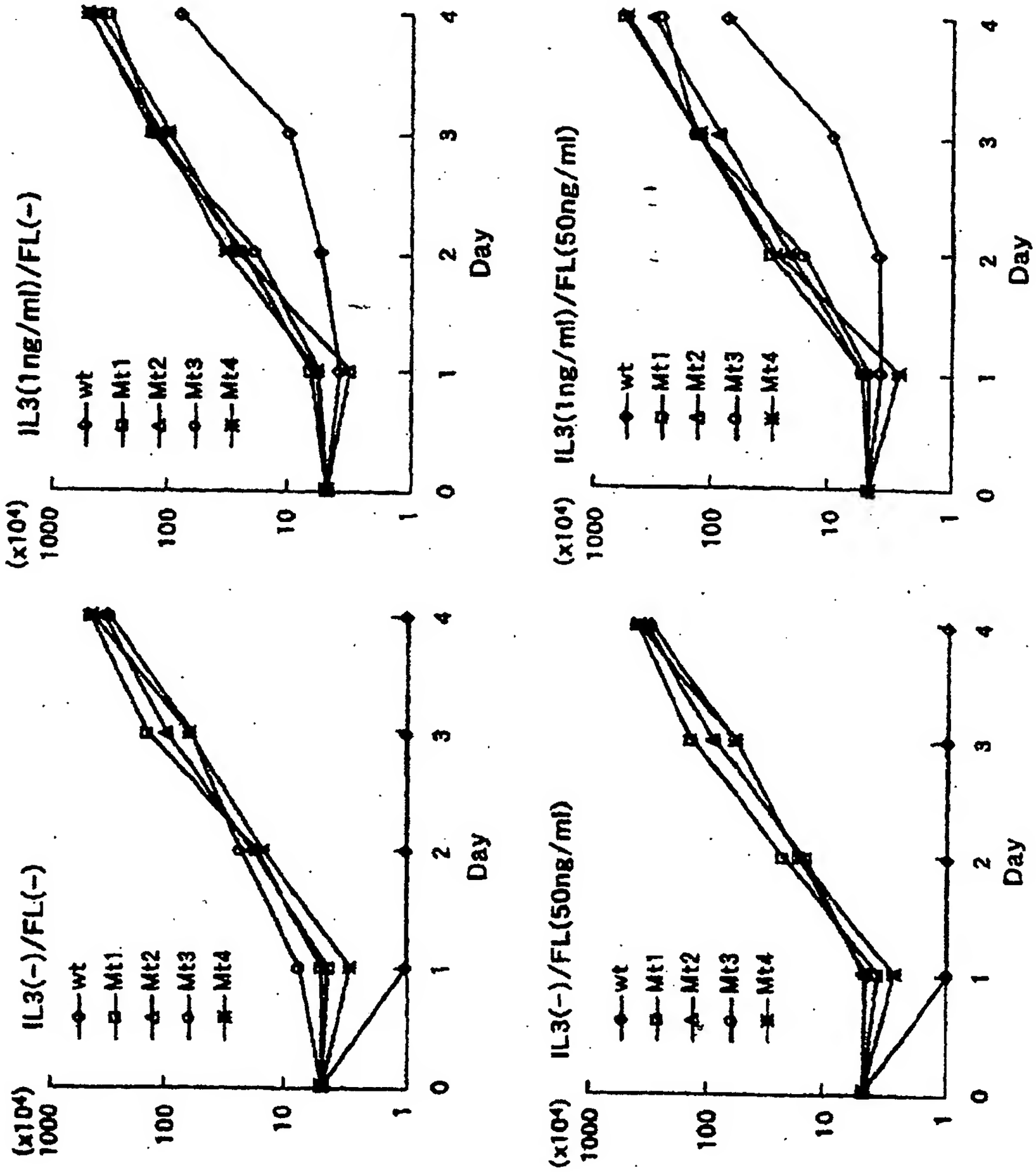
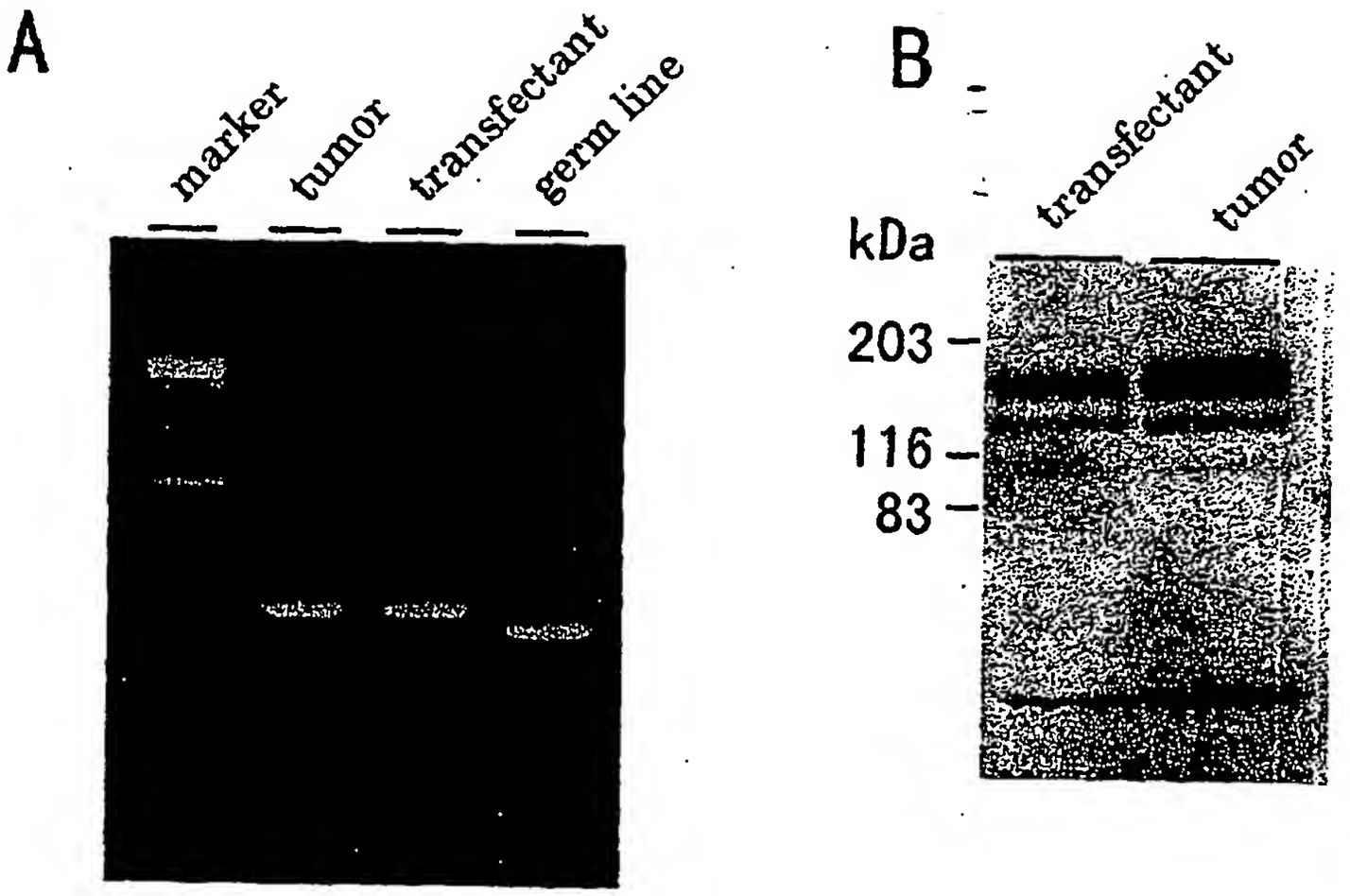


Fig. 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP99/04450

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[6] G01N33/50, 33/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[6] G01N33/50, 33/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Toroku Jitsuyo Shinan Koho 1994-1999
Kokai Jitsuyo Shinan Koho 1971-1999 Jitsuyo Shinan Toroku Koho 1996-1999

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS [internal tandem duplication? * FLT3]

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. Yokota, "INTERNAL TANDEM DUPLICATION OF THE FLT3 GENE IS PREFERENTIALLY SEEN IN ACUTE MYELOID LEUKEMIA AND MYELODYSPL ASTIC SYNDROME AMONG VARIOUS HEMATOLOGICAL MALIGNANCIES. A STUDY ON A LARGE SERIES OF PATIENT AND CELL LINES" LEUKEMIA, VOL. 11, (1997), p.1605-1609	1-10
A	M. Nakao, "INTERNAL TANDEM DUPLICATION OF THE FLT3 GENE FOUND IN ACUTE MYELOID LEUKEMIA", LEUKEMIA, VOL. 10, (1996), p.1911-1918	1-10
A	H. Kiyoi, "INTERNAL TANDEM DUPLICATION OF FLT3 ASSOCIATED WITH LEUKOCYTOSIS IN ACUTE PROMYELOCYTIC LEUKEMIA" LEUKEMIA, VOL. 11, (1997), p.1447-1452	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 September, 1999 (29. 09. 99)	12 October, 1999 (12. 10. 99)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP99/04450

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Although the invention as set forth in claim 11 relates to candidate compounds for a drug, it is unclear which compounds are involved therein.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

XP-002272706

AN - PREV200200220615

TI - Changes in the pattern of FLT3 mutations between diagnosis and relapse restricts the potential use as markers of minimal residual disease in patients with acute myeloid leukaemia

AB - Although approximately 80% of adult patients less than 55 years of age with acute myeloid leukaemia (AML) will achieve complete remission (CR), the long term survival still only about 40% and therefore attention has been increasingly focussed on identifying factors that predict for relapse. Several recent studies have shown that an internal tandem duplication (ITD) in the juxtamembrane domain of the tyrosine kinase receptor FLT3 is a frequent mutation in AML and adversely predicts for overall surviva and relapse risk (RR). In our own study of 854 patients less than 60 years of age, 27% had a FLT3/ITD (ITD+). Multivariate analysis showed that it was the most significant factor predicting for RR and it added important prognostic information to cytogenetic risk group. More recently, activating point mutations at residue D835 of FLT3 have been identified in 7% of AML patients, indicating that approximately one third of all cases of adult AML have mutations of the FLT3 gene. In order to determine whether these FLT3 mutation could be used as a marker of minimal residual disease (MRD), w studied paired DNA samples from patients at different stages of their disease. All patients had de novo AML and were treated according to the UK MRC AML 10 and 12 trial protocols. Thirteen patients who were ITD+ at diagnosis, median mutant level 44% (range 2% to 90%), were all ITD- at CR. One patient also had a D835 mutation at diagnosis (D835+) which was not present in CR. These results confirm that the FLT3 mutations had been acquired as part of the disease process. Forty four patients were then studied at diagnosis and relapseTwenty four patients had only wild type FLT3 at diagnosis. Four of these acquired a FLT3 mutation at 1st relapse: 2 became ITD+ (mutant levels 38% and 42%) and 2 heterozygous D835+. One further patient became ITD+ at 2nd relapse (45% mutant). Twenty patients had a FLT3 mutation at diagnosis (18 ITD+ and 2 D835+), five of them lost their ITD at relapse. Their mutant levels at presentation were 6%, 11%, 20%, 28% and 44% respectively, indicating that in at leas 3 of these patients the mutant clone had originally comprised a significant proportion c the total cells. In 14 of the 15 patients who had a mutation at presentation and relapse sequencing and quantification showed that 8 of these patients relapsed with the same mutation as at diagnosis at approximately the same levelTwo were heterozygous D835+, 6 were ITD+ with median difference in mutant level between diagnosis and relapse of 0% (range -3% to 3%). One of the latter patients became D835+ at relapse. Six ITD+ patients had the same mutation at diagnosis as relapse but at an increased level (median difference 29.5%, range 13-65%). In one of these patients 3 mutants were present at diagnosis, but only the predominant mutant was detected at relapse. The remaining patient was ITD+ at diagnosis and relapse but had 2 completely differe mutations. Three polymorphic markers were studied in the paired samples from all patients with a different pattern at diagnosis and relapse to confirm patient identity. These results indicate that although the majority of patients have the same FLT3 statu at diagnosis and relapse, FLT3 mutations can be lost or acquired at relapse and therefore they are not suitable as markers of MRD.

IW - ** Major Concepts **
Blood and Lymphatics (Transport and Circulation) ;Immune System (Chemical Coordination and Homeostasis) ;Molecular Genetics (Biochemistry and Molecular Biophysics) ;Tumor Biology